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Calof AL, et al
Regulation of neurogenesis and neuronal differentiation in primary and immortalized cells from mouse olfactory epithelium.
Ciba Found Symp. 1991;160:249-65; discussion 265-76.
PMID: 1752166; UI: 92090184.

Pixley SK, et al.
Cultured rat olfactory neurons are excitable and respond to odors.
Brain Res Dev Brain Res. 1990 Apr 1;53(1):125-30.
PMID: 2350878; UI: 90275756.

Pixley SK.
Characterization of olfactory receptor neurons and other cell types in dissociated rat olfactory cell cultures.
Int J Dev Neurosci. 1996 Nov;14(7-8):823-39.
PMID: 9010728; UI: 97163981.

NO
11/30

Noble M, et al.
The growth of olfactory neurons in short-term cultures of rat olfactory epithelium.
Neurosci Lett. 1984 Mar 23;45(2):193-8.
PMID: 6728313; UI: 84220439.

Maue RA, et al.
Preparation of isolated mouse olfactory receptor neurons.
Pflugers Arch. 1987 Jul;409(3):244-50.
PMID: 3627945; UI: 87316795.

Schubert D, et al.
Ontogeny of electrically excitable cells in cultured olfactory epithelium.
Proc Natl Acad Sci U S A. 1985 Nov;82(22):7782-6.
PMID: 2866512; UI: 86067989.

Wolozin B, et al.
Continuous culture of neuronal cells from adult human olfactory epithelium.
J Mol Neurosci. 1992;3(3):137-46.
PMID: 1320921; UI: 92329300.

Sosnowski JS, et al.
Chemical traumatization of adult mouse olfactory epithelium in situ stimulates growth and differentiation of olfactory neurons in vitro.
Brain Res. 1995 Dec 8;702(1-2):37-48.
PMID: 8846094; UI: 96255746.

Eustache I, et al.
Effects of insulin-like growth factors on organotypic cocultures of embryonic rat brainstem slices and skeletal muscle fibers.
Brain Res Dev Brain Res. 1994 Sep 16;81(2):284-92.
PMID: 7813048; UI: 95112382.

Developmental Brain Research, 53 (1990) 125–130
Elsevier

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Cultured rat olfactory neurons are excitable and respond to odors

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Newborn rat nasal tissues containing olfactory epithelium were dissociated and maintained in a monolayer cell culture. Neurons were present, as determined by immunostaining with antibodies to 4 neuron-specific proteins: neuron-specific enolase, microtubule-associated protein 2, tau protein and synaptophysin. Immunostained neurons had a distinctive morphology resembling olfactory neurons. By patch-clamp analysis, these cells were electrically active. Responses of some neurons to physiological concentrations of an odorant mixture identified them as olfactory receptor cells.

Olfactory receptor neurons (ORNs) pose two challenges to experimenters: they are the only mammalian neurons which undergo cellular turnover and replacement in the adult[10] and the initial events of olfactory transduction are not known[1]. In vitro systems of ORNs would greatly facilitate studies of both phenomena because of improved accessibility of the cells. Previously described primary monolayer ORN culture systems[8,18,20] have not been widely used, primarily for two reasons. First, ORNs are difficult to identify in culture since the ORN neurotransmitter is not known (carnosine is a candidate) and ORNs do not contain neurofilaments. Second, ORN survival has been poor in monolayers, requiring feeder layer cells[18], or resulting in low neuronal yields[20]. This may result from a high sensitivity of ORNs to disaggregation[12]. We present a monolayer culture system of olfactory tissue which allows excellent survival of isolated neurons which were electrically active and showed responses to odors.

Anesthetized, newborn Spraque–Dawley rat pups were decapitated, the nasal tissues were removed, minced, incubated for 1 h in 0.0625% trypsin and 0.175 mg/ml collagenase (Sigma, St. Louis, MO) and mechanically dissociated. Viabilities were >90%, yields approximately 4×10^5 cells/pup. Cells were plated onto NaOH-treated (10 N for 20 min, rinsed), polylysine-coated (0.05 mg/ml, Sigma no. P-7890, overnight) glass coverslips (10 or 12 mm rounds, Dynalab, Rochester, NY) in 48 or 24 multiwell dishes (Gibco), respectively. Laminin coating (10 μg/ml, 3-h incubation after polylysine) did not change neuron survival or morphology. Cells were maintained at 100% humidity in air/5% CO_2, at 37 °C, with the first media change at 4 days. The culture medium was essentially the hypoxanthine-thymidine-containing, hybridoma cell maintenance medium[2], except for the use of Dulbecco's minimum essential medium (DMEM, Whittaker M.A. Bioproducts, Walkersville, MD, high glucose, bicarbonate, no glutamine) as the basal medium, with the addition of 18 mM HEPES, 4 mM glutamine, 1× non-essential amino acids (Sigma), 10% heat-inactivated horse serum (J.R. Scientific, Woodland, CA) and 10% fetal calf serum (Hyclone, Logan, UT). Dissociated cells from newborn rat nasal tissues were plated at low density (1.5×10^5 cells/cm^2) for short-term survival. Several cell morphologies were observed. Before culture confluency was reached (about 1 week after plating), neuron-like cells with processes were seen attached directly to the substrate, often without contacting other cells (Fig. 1).

Four neuron-specific antibodies were used to identify neurons in culture. Recently, it has been shown that antibodies to neuron-specific enolase (NSE)[5], microtubule-associated protein-2 (MAP2)[23] and tau protein[23] bind specifically to ORNs in the intact olfactory epithelium. We have immunostained adult rat nasal tissue sections with antiserum to synaptophysin, a packaging protein unique to synaptic vesicles of neurons and neuroendocrine cells[3] and observed specific staining of olfactory nerve bundles in the lamina propria and ORN dendrites in the olfactory epithelium (not shown). In

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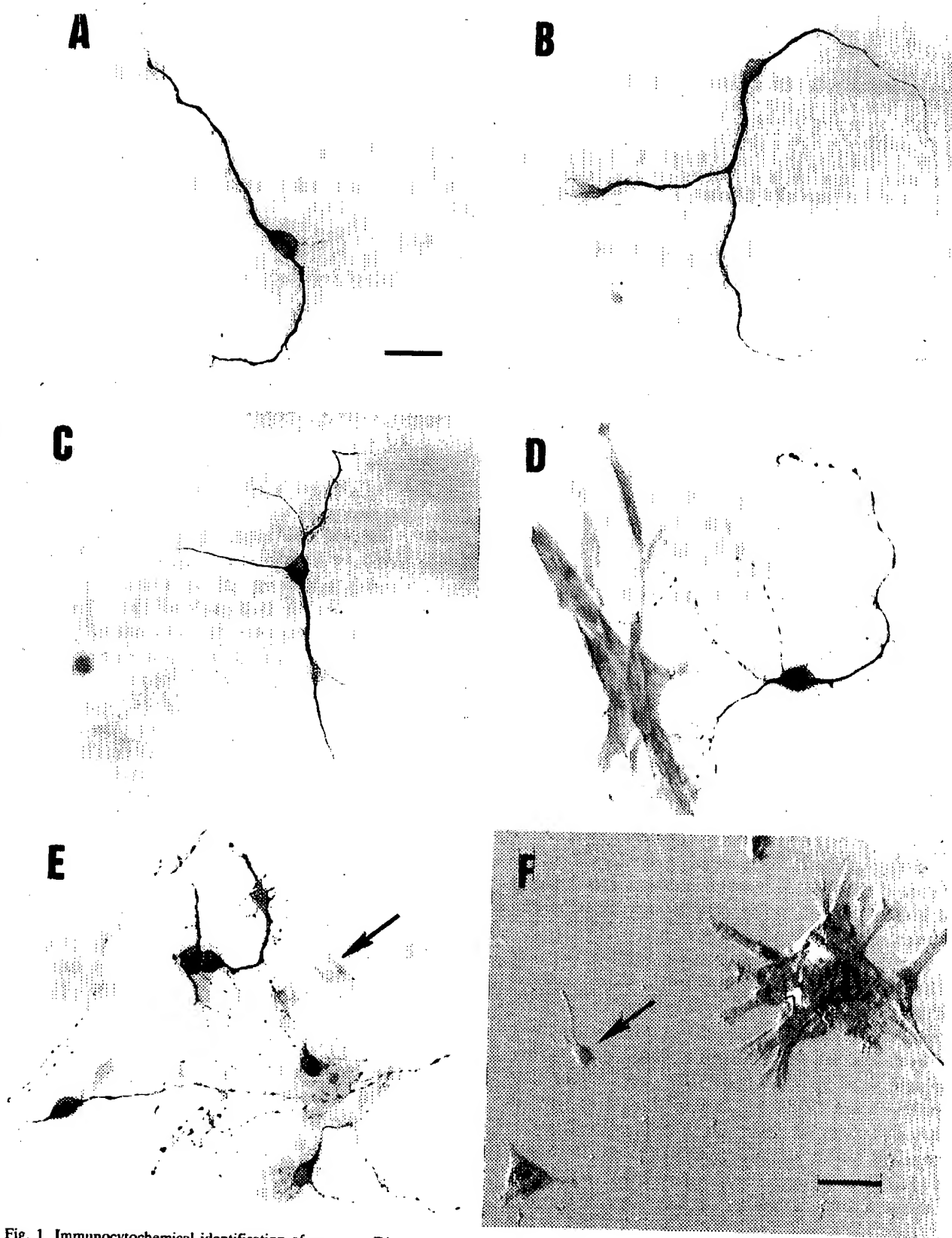

Fig. 1. Immunocytochemical identification of neurons. Dispersed newborn rat nasal cells cultured for 4 days contained cells with neuronal morphology which were positive for MAP2 (A), tau (B), synaptophysin (C and E) and NSE (D). Cells were plated at low density (A–D and F) or high density (a cluster of neurons is shown in E). The arrow in E shows the position of an unstained non-neuron. Neuron-like cells were negative for fibronectin (F, arrow). Bar = 15 μm (A–E) or 30 μm (F).

culture, cells separately immunostained with each of these antibodies had a distinctive, neuronal morphology (Fig. 1A–E). For most immunocytochemistry, cells were fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer), pH 7.4, for 15 min at room temperature. For immunostaining with anti-NSE, cells were fixed in: 4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% saturated picric acid in 0.1 M sodium acetate buffer, pH 6.0[5]. Cells were incubated (1 h) with primary antibodies containing 0.2% Triton X-100, followed by application of the appropriate avidin-biotin-immunoperoxidase Vectakit (Vector Labs, Burlingame, MD) using diaminobenzidine as the chromagen and glucose oxidase to generate H_2O_2. Dilutions were: anti-NSE, 1/2000 (rabbit polyclonal, Polysciences, Warrington, PA); anti-MAP2 (AP14), 1/500; anti-tau, 1/250 (mouse monoclonals, gifts of L. Binder[22]) and anti-synaptophysin, 1/250 (mouse monoclonal anti-SV2, gift of R. Kelly[3]), anti-fibronectin, 1/1000 (rabbit polyclonal, DAKO, Carpinteria, CA) anti-S100, 1/1000 (rabbit polyclonal, DAKO) and anti-laminin, 1/1000 (Collaborative Res., Bedford, MA). Non-neurons were negative for MAP2, tau or synap-

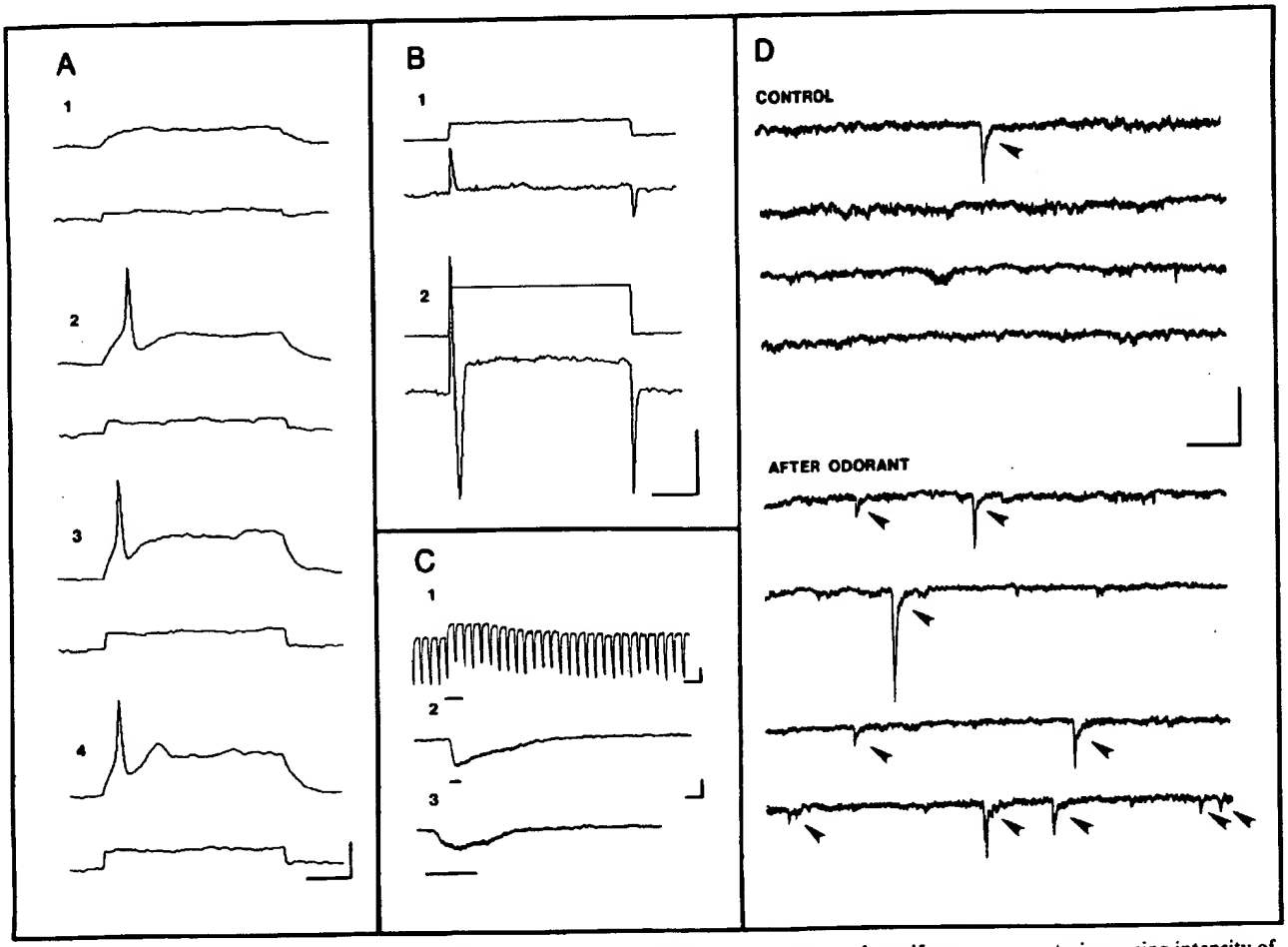

Fig. 2. Electrophysiological responses of cultured olfactory neurons. A: membrane responses of an olfactory neuron to increasing intensity of stimulation. Top traces indicate membrane potential; bottom traces indicate current injected. A depolarizing current pulse, duration 200 ms and intensity 0.01 nA (1), did not elicit an action potential. Increasing the stimulus intensity to 0.02 nA (2) evoked an AP which was followed by an after-hyperpolarization. Note the faster rising phase at higher stimulating current, 0.03 (3) and 0.04 nA (4). Cell membrane potential was –53 mV. Calibration: 22 mV, 80 pA, 50 ms. B: an olfactory neuron under voltage-clamp conditions. Bottom traces indicate membrane current as recorded (i.e. leakage and capacitative currents were not subtracted); upper traces indicate voltage steps. A depolarizing step, duration 100 ms, to –50 mV (1) from a holding potential of –60 mV did not elicit any inward current. Depolarizing to –30 mV (2) evoked a fast inward current, followed by an outward current. Calibration: 40 mV, 45 pA, 25 ms. C: response of an olfactory neuron to an odorant mixture. Application of odorants produced a depolarization accompanied by an increase in conductance of the membrane (1). Under voltage-clamp conditions, odorant application evoked an inward current in the same cell (2). In another cell, the inward current elicited by the odorous material started to decrease before the application had terminated, suggesting that desensitization may have occurred. Note the increase in current noise during odorant responses (2,3). Calibration: 1: 20 mV, 2 s; 2–3: 200 pA, 2 s. D: activation of transient currents by odorants. Membrane current was measured in an olfactory neuron voltage-clamped at a potential of –80 mV. The frequency of occurrence of transient currents, which resemble synaptic currents (arrowheads) was very low (Control). Application of odorant enhanced the frequency of appearance of transient currents (After odorant). Calibration: 100 pA, 600 ms.

tophysin (Fig. 1E, arrow), but showed light staining with anti-NSE (Fig. 1D). Antibody controls, which were negative, were the use of normal rabbit serum instead of anti-NSE, or omission of primary antibody instead of the mouse monoclonal antibodies. Cells with neuronal morphology did not immunostain for fibronectin (Fig. 1F), S100 or laminin, indicating that they were neither fibroblasts, Schwann/glial cells nor perineurial cells, respectively. Cell morphology was compared with immunostaining using differential interference contrast optics (20 fields per coverslip, 5 coverslips (from 3 experiments) per antibody). A small percentage of all cells with neuronal morphology were immunonegative no matter which neuron-specific antiserum was used (13.6 ± 1.5% mean ± S.E.M., n = 20 coverslips, or 13.6 ± 1.3%, n = 4 antibodies). Both NSE[19] and synaptophysin staining suggest some degree of functional maturity of neurons, so the unstained cells with neuronal morphology may be immature neurons.

Olfactory marker protein (OMP) is made specifically by mature ORNs in the intact olfactory epithelium. OMP-positive cells have been detected in olfactory explants[8], but not monolayer cultures[8,18,20]. Using two rabbit (R. Reed) and one goat antisera (F. Margolis), we found no OMP-positive cultured cells. This may suggest immaturity of the neurons, although the presence of synaptophysin, NSE and the electrophysiological characteristics (see below) would suggest otherwise. Additional factors must be required for expression of immunologically detectable levels of OMP. This culture system allows for environmental control, which might lead to determination of such factor(s).

Immunostained neurons had small, elliptical cell bodies which averaged 14.6 ± 0.4 by 10.4 ± 0.3 μm (mean ± S.E.M., n = 30). Compared to non-neurons, they had less cytoplasm, more abrupt cell body-to-process transitions and longer, thinner processes. The majority of cultured neurons had two processes longer than one cell body diameter (67%, n = 322), with 22% having three. MAP2 or tau immunostaining was not restricted to either dendrite-like processes (variable width and curved), or axon-like processes (narrower, invariant in diameter and straight), as has been observed in other neuronal culture systems. ORNs in vivo are bipolar with one axon and dendrite, both generally considered unbranched, although one report describes dendritic branching[9]. Cultured neurons resembled tissue ORNs in being bipolar, but differed by having process branching. This suggests that the ORN shape may be determined by both intrinsic and extrinsic components.

Counts of NSE-positive neurons, 4 days after plating, averaged 1240 ± 143 neurons/cm^2 (mean ± S.E.M., n = 16 coverslips, 10 random microscope fields (0.025 cm^2/field) counted per coverslip, 5 separate experiments). Counts did not vary with the other antisera. Neurons survived 1–2 weeks in low-density cultures, but as long as 25 days (longest time examined) if a different dissociation technique and higher plating density (4×10^5 cells/cm^2) were used. For long-term survival, the dissociating enzyme concentrations were increased 2-fold and there were four 20-min enzyme incubation/mechanical dissociation steps instead of one 1-h step. Media changes were every 4–6 days. In high-density cultures, non-neurons formed a confluent bed layer under neurons after only 4 days (Fig. 1E), impeding electrophysiological and morphological observations. Odorant responses and electrophysiological measurements have not been done on long-term cultures.

Neurogenesis was not observed in these cultures. Neuron numbers did not increase during the first 4 days in culture. Tritiated thymidine (Amersham, Arlington Heights, IL, 2 μCi/ml) was added one day after plating, for 12–18 h, then replaced with fresh media. Coverslips were fixed immediately and every day for the next 4 days. Anti-NSE immunoperoxidase staining was followed by standard liquid emulsion autoradiography (Kodak NTB-2 emulsion coating (1:1 in water), developed with D-19 developer (1:1) and Rapid Fix after 1 week exposure at 4 °C). In three 4-day experiments, 2–3 coverslips per day, 10 microscope fields per coverslip were examined. No silver grains were found associated with immunostained neurons (not shown). Calof and Chikaraishi have demonstrated neurogenesis in olfactory cell cultures from embryonic mice during the same time period in culture[4], so our observations may reflect differences in culture conditions or animal age.

Electrophysiological studies were performed on cells after 3–5 days in culture using the whole-cell, patch-clamp recording technique. An Axoclamp 2A amplifier (Axon Instruments, Burlingame, CA) was used to record from cells bathed in either a nutrient medium supplemented with 25 mM HEPES, or a HEPES-buffered medium (mM): NaCl 140, KCl 4, $CaCl_2$ 3, $MgCl_2$ 1, glucose 10, HEPES 10, pH 7.3–7.4, osmolarity 325 mOs. Similar results were obtained from the two different recording solutions. The recording pipette solution contained (mM): KCl 140, $MgCl_2$ 2, EGTA 1.1, glucose 10, HEPES 10, pH 7.1, osmolarity 315 mOs. Resistances of the electrodes were between 12 and 16 Mohm.

Neurons, identified morphologically, were electrically excitable in response to current injection, i.e. an action potential (AP) could be evoked. Successful recordings were made mainly from single, isolated cells (n > 50). Neurons had a membrane potential of –50 mV (mean –50 ± 2 (S.E.M.) mV; range –40 to –65 mV, n = 16) and an input resistance of 5.3 ± 1.0 Gohm (mean ± S.E.M.,

n = 11) (range 1.5–10.6 Gohm). One additional cell had an exceptionally high input resistance of 15 Gohm and was excluded from the calculations. These values are in the range of those reported for salamander olfactory neurons[6,7,11,14,16,21] and dissociated rodent olfactory neurons[13,15]. The threshold for AP generation was between 0.02 and 0.05 nA, also in the range of that reported for dissociated and regenerating salamander neurons[6,7,11,14,16,21]. An example of an evoked AP is shown in Fig. 2A. Cells which did not show an evoked AP usually had low membrane potential and poor seal resistance. Although repetitive firing to long current pulses has been reported in salamander ORNs[11,14], we have not systematically investigated the effects of injecting large amplitude currents for long durations.

Under voltage-clamp conditions, a fast inward current followed by an outward current was elicited by depolarizing steps (> +30 mV) from a holding potential of –60 mV, or more negative. An example is shown in Fig. 2B. Similar current patterns have been described in other ORN preparations. The ionic currents underlying the AP in our cells will be described in detail elsewhere (R. Pun, in preparation).

Other cell types present in culture had lower membrane potentials (<–30 mV, n = 15) and were not electrically excitable, except for cells which were oval in morphology, larger than neurons and found mainly in clumps. These cells had slow AP-like waveforms upon injection of depolarizing currents >1 nA and showed pronounced inward rectification. These membrane characteristics resemble those of human astrocytoma cells (R. Pun, unpublished observations) and Muller cells[17], suggesting that these may be sustentacular or glial cells. Cells of this morphology did not immunostain with any neuron-specific antiserum.

Neurons responded to odorants added to the bathing medium. The stimulant, containing 10 different odorants, each at a concentration of 1 μM, was applied by pressure from a blunt-tipped micropipette (3–5 μm diameter) placed 30–50 μm from the cell. The odorant mixture contained: 2-heptanone, (*S*)(+)-carvone, isoamyl acetate, anisole, pyridine, benzaldehyde, hexanoic acid, cineole, *n*-butanol and ethyl *n*-butyrate. Of 10 cells showing evoked APs, 5 responded with a depolarization, which was associated with an increase in membrane conductance (Fig. 2C, 1). Cells did not respond to media similarly applied. Under voltage-clamp conditions, odorant application produced an inward current (Fig. 2C, 2, 3). In some cells, spontaneous events increased with odorant application (Fig. 2D, arrows). The origin and ionic mechanisms generating these phenomena are not known. The slow inward current, which outlasted the application of the stimulant is similar to the odorant response in dissociated salamander olfactory neurons[7], although the odorant concentrations used were higher (5 odorants, each at 0.1–1 mM). The percentage of responding cultured cells is high compared to the 25% observed in freshly dissociated mouse ORNs[15]. This may reflect differences between dissociated and cultured cells, or differential viability of cell types in culture. This may also reflect the high yield and good viability of differentiated neurons in our culture system. We believe that this culture system will be very useful for studies of sensory transduction mechanisms, intrinsic morphological properties of ORNs and identification of neurotrophic or neurogenic substances specific for ORNs.

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1 Anholt, R.R.H., Primary events in olfactory reception, *Trends Biol. Sci.*, 12 (1987) 58–62.
2 Boss, B., An improved in vitro immunization procedure for the production of monoclonal antibodies, *Methods Enzymol.*, 121 (1986) 27–33.
3 Buckley, K. and Kelly, R.B., Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells, *J. Cell Biol.*, 100 (1985) 1284–1294.
4 Calof, A. and Chikaraishi, D., Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro, *Neuron*, 3 (1989) 115–127.
5 Coon, H.G., Curcio, F., Sakaguchi, K., Brandi, M.L. and Swerdlow, R.D., Cell cultures of neuroblasta from rat olfactory epithelium that show odorant responses, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 1703–1707.
6 Firestein, S. and Werblin, F., Gated currents in isolated olfactory receptor neurons of the larval tiger salamander, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 6292–6296.
7 Firestein, S. and Werblin, F., Odor-induced membrane currents in vertebrate-olfactory receptor neurons, *Science*, 244 (1989) 79–82.
8 Gonzales, F., Farbman, A. and Gesteland, R.C., Cell and explant culture of olfactory chemoreceptor cells, *J. Neurosci. Methods*, 14 (1985) 77–90.
9 Graziadei, P.P.C., Topological relations between olfactory neurons, *Z. Zellforsch.*, 118 (1971) 449–466.
10 Graziadei, P.P.C. and Monti Graziadei, G.A., The olfactory system: a model for the study of neurogenesis and axon regeneration in mammals. In C.W. Cotman (Ed.), *Neuronal Plasticity*, Raven, New York, 1978, pp. 131–153.
11 Hedlund, B., Masukawa, L.M. and Shepherd, G., Excitable properties of olfactory receptor neurons, *J. Neurosci.*, 7 (1987) 2338–2343.
12 Hirsch, J.D. and Margolis, F.L., Cell suspensions from rat olfactory neuroepithelium: biochemical and histochemical characterization, *Brain Research*, 1 (1979) 277–291.
13 Lynch, J.W. and Barry, P.H., Action potentials initiated by single channels opening in a small neuron (rat olfactory receptor), *Biophys. J.*, 55 (1989) 755–768.
14 Masukawa, L.M., Hedlund, B. and Shepherd, G.M., Electrophysiological properties of identified cells in the in vitro

olfactory epithelium of the tiger salamander, *J. Neurosci.*, 5 (1985) 128–135.

15 Maue, R. and Dionne, V., Patch-clamp studies of isolated mouse olfactory receptor neurons, *J. Gen. Physiol.*, 90 (1987) 95–125.

16 Maue, R. and Dionne, V., Preparation of isolated mouse olfactory receptor neurons, *Pflügers Arch.*, 409 (1987) 244–250.

17 Newman, E.A., Voltage-dependent calcium and potassium channels in retinal glial cells, *Nature (Lond.)*, 317 (1985) 805–811.

18 Noble, M., Mallaburn, P.S. and Klein, N., The growth of olfactory neurons in short-term cultures of rat olfactory epithelium, *Neurosci. Lett.*, 45 (1984) 193–198.

19 Schmechel, D.E., Brightman, M.W. and Marangos, P.J., Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation, *Brain Research*, 190 (1980) 195–214.

20 Schubert, D., Stallcup, W., LaCorbiere, M., Kidokoro, Y. and Orgel, L., Ontogeny of electrically excitable cells in cultured olfactory epithelium, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 7782–7786.

21 Trotier, D., A patch-clamp analysis of membrane currents in salamander olfactory receptor cells, *Pflügers Arch.*, 407 (1987) 589–595.

22 Tucker, R.P., Binder, L.I. and Matus, A.I., Neuronal microtubule-associated proteins in the embryonic avian spinal cord, *J. Comp. Neurol.*, 271 (1988) 44–55.

23 Viereck, C., Tucker, R.P. and Matus, A., The adult olfactory system expresses juvenile forms of microtubule-associated proteins, *Soc. Neurosci. Abstr.*, 14 (1988) 1041.